10/507405

PCT/GB 2003 / 001375

Rec'd PCT/PTO 10 SEP 2004

The Patent Office

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

REC'D 19 MAY 2003
WIPO PCT

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

BEST AVAILABLE COPY

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Signed Stephen Hordley

Dated 7 April 2003

Patents Form 1/77

Patents Act 1977
(Rule 16)

THE PATENT OFFICE
02 APR 2002
RECEIVED BY HAND

The Patent Office

1/77

03APR02 E708116-5 D02896
P01/7700 0.00-0207644.6

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
South Wales
NP9 1RH

1.	Your reference	REP06595GB
2.	Patent application number *(The Patent Office will fill in this part)*	02 APR 2002 0207644.6
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	Ark Therapeutics Ltd. 1 Fitzroy Mews London W1T 6DE
	08100612003 Patents ADP number *(if you know it)*	
	If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom
4.	Title of the invention	PEPTIDES AND THEIR USE
5.	Name of your agent *(if you have one)*	Gill Jennings & Every
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	Broadgate House 7 Eldon Street London EC2M 7LH
	Patents ADP number *(if you know it)*	745002 ✓
6.	If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country Priority application number *(if you know it)* Date of filing *(day / month / year)*
7.	If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application Date of filing *(day / month / year)*
8.	Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:* *a) any applicant named in part 3 is not an inventor, or* *b) there is an inventor who is not named as an applicant, or* *c) any named applicant is a corporate body.* *See note (d))*	YES

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	6
Claim(s)	1
Abstract	
Drawing(s)	2

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	NO

11. For the applicant
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

Signature

Date
2 April 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

R E Perry
020 7377 1377

Warning
After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes
a) *If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.*
b) *Write your answers in capital letters using black ink or you may type them.*
c) *If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.*
d) *If you have answered 'Yes' Patents Form 7/77 will need to be filed.*
e) *Once you have filled in the form you must remember to sign and date it.*
f) *For details of the fee and ways to pay please contact the Patent Office.*

PEPTIDES AND THEIR USE

Field of the Invention

This invention relates to peptides which have activity of potential benefit in therapy.

Background of the Invention

VEGF is a secreted polypeptide which is essential for formation of the vascular system in embryogenesis and plays a major role in angiogenesis in a variety of disease states. VEGF expression is upregulated by hypoxia and several cytokines in diverse cell types, and elicits multiple biological activities in vivo and in vitro including the differentiation, proliferation, migration and survival of endothelial cells, increased vascular permeability, monocyte migration, and increased endothelial production of the vasodilatory factors NO and prostacyclin. VEGF-induced NO and prostacyclin production are in turn implicated in both angiogenesis and several vascular protective effects of VEGF, including increased permeability, and inhibition of intimal vascular smooth muscle cell hyperplasia and thrombosis.

Human VEGF exists in multiple isoforms of 121, 145, 165, 189 and 206 amino acids, generated by alternative mRNA splicing, of which $VEGF_{121}$, $VEGF_{145}$ and $VEGF_{165}$ are known to be secreted and biologically active forms. Two distinct protein tyrosine kinase receptors for VEGF have been identified, Flt-1 (VEGFR1) and KDR/Flk-1 (VEGFR2). KDR/flk-1 is thought to be the receptor which primarily mediates the mitogenic effects of VEGF in endothelial cells and angiogenesis in vivo; the function of Flt-1 in endothelial cells is unknown. A non-tyrosine kinase transmembrane protein, neuropilin-1 (NP-1), has been identified as an additional receptor for VEGF which specifically binds $VEGF_{165}$, and enhances binding of $VEGF_{165}$ to VEGFR2. The role of NP-1 in mediating biological effects of VEGF is still largely unknown.

NP-1 is a receptor for a family of molecules called semaphorins or collapsins which play a key role in the guidance of neuronal axons during mammalian development. In particular, NP-1 is known to mediate the growth cone-collapsing and chemorepulsive activity of semaphorin 3.

Soker *et al*, J. Biol. Chem. 271(10): 5761-5767 (1996), discloses that a GST fusion protein containing the 44 amino acids encoded by exon 7 bind to NP-1.

Soker *et al*, J. Biol. Chem. 272(10): 31582-31588 (1997), discloses that a 23 amino acid region of exon 7 is necessary for inhibition of VEGF binding to HUVECs. The shortest active peptide is

CSCKNTDSRCKARQLELNERTCRC

i.e. VEGF (139-160), or amino acids 22-44 of exon 7 and amino acid 1 of exon 8. The terminal cysteine residue (C^{137} in VEGF) is apparently essential for activity and the molecule's 3D structure. It is suggested that there may be intradisulfide bonding within the VEGF monomer.

Summary of the Invention

Surprisingly, it has been found that contain novel peptides have NP-1 antagonist activity.

According to the present invention, a peptide has the amino acid sequence

SCKNTDSRCKARQLELNERTCRCDKPRR

or a fragment thereof that substantially retains NP-1 antagonist activity, in cyclic form.

The sequence corresponds to amino acids 138 to 165 within VEGF, i.e. including part at least of exon 8. The invention also encompasses variants of this sequence, in which the novel activity, i.e. the NP-1 antagonism, is retained without unexpected structural variation. Thus, the given sequence may include isosteric or homologous replacements or derivatisation that renders the peptide relatively stable.

Description of Preferred Embodiments

Peptides of the invention may be synthesised by known methods. Examples are given below. They may be formulated and used in known manner. The anti-angiogenic activity associated with the peptides means that they can be used in the treatment of tumours.

A peptide of this invention preferably has 4 Cys residues. Such a peptide may be cyclised, by known means; see, for example, Tam *et al*, JACS 113:6657-

62 (1991). Other cyclisations, e.g. Mitsunobu or olefin metathesis ring closure, may also be used. The cyclic peptides may exhibit enhanced properties.

As indicated above, peptides of the invention include modifications of the given sequence. Such modifications are well known to those skilled in the art. Isosteric replacements include Abu for Cys (this may be desirable where the peptide should retain an even number of Cys residues for cyclisation), Phe for Tyr and different alkyl/aryl substituents. The shifting of substituents within an amino acid residue, from a C atom to a N atom, to produce peptoids having greater resistance to proteolysis, and other modifications, are known and are included within the scope of this invention. The specific peptide reported here is N-acetylated; other terminal modifications will also be known to those of ordinary skill in the art.

The NP-1 antagonist properties of a peptide of this invention may be determined by the procedure described below. The level of activity is preferably at least 25 or 50% as great as that for the bicyclic 28-mer that has been synthesised.

Peptides of the invention may be formulated and administered by procedures, and using components, known to those of ordinary skill in the art. Their activity means that they may be useful in the treatment of diseases in which NP-1 may have a significant role in pathology.

NP-1 antagonist may compete with semaphorin 3 for binding to NP-1, and thereby antagonise effects of semaphorin 3 on axonal outgrowth and migration in nerve cells. Potential applications of this are in promoting neurite outgrowth, in stimulating nerve repair or treating neurodegeneration. Further, an NP-1 antagonist may promote the survival of semaphorin 3-responsive neurones, an effect that would strengthen its use in the applications given above, and may extend these applications to, e.g. treating neuronal death caused by episodes of ischaemia as in stroke and some eye diseases.

Peptide Syntheses

All peptides were synthesised on an automated AMS 422 Multiple Peptide Synthesiser using the solid phase approach. The Rink Amide MBHA resin (0.59 and 0.68 mmol/g loading) and the N-Fmoc strategy with orthogonal protection

(Acm, t-Bu) of the Cys side chains of derivatives to be cyclised were applied. The desired peptide was synthesised on a 25 μM scale and coupled once with a basis coupling time of 30 minutes. The resin and the amino acid derivatives, Fmoc-Ala-OH.H_2O, Fmoc-Arg(Pbf/Pmc)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro.H_2O, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH and Fmoc-Val-OH were purchased from Calbiochem-Novabiochem UK Ltd. (Nottingham, UK) or Alexis (Nottingham, UK).

Each amino acid was sequentially coupled to the growing peptide chain from the C- to the N-terminus applying benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluoro-phosphate (Pybop, Calbiochem-Novabiochem) and N-methyl morpholine (NMM, Rathburn Chemicals, Walkerburn, UK) as coupling reagents *via* the active ester method. Removal of the N-Fmoc protecting group was carried out with 20 % piperidine in DMF (Rathburn Chemicals, Walkerburn, Scotland) followed by sequential washes with DMF and DCM. Automatic acetylation was carried out after the synthesis of each peptide with a 4-fold excess of acetic acid (0.7 molar, Rathburn Chemicals, Winterburn Scotland) based on the substitution of the Rink-Amide-MBHA resin. The coupling reagent, Pybop, NMM and all amino acid derivatives were dissolved in DMF (0.7 M, 4-fold excess based on the substitution of the Rink-Amide-MBHA-resin) except for the amino acids Fmoc-His(Trt)-OH and Fmoc-Phe-OH. These protected amino acid derivatives were dissolved in N-methylpyrrolidone. All solvents used were of HPLC-grade quality. The peptides were cleaved from the resin with simultaneous deprotection using 90 % TFA at room temperature for 3 hours in the presence of 5 % thioanisole, 2.5 % water and 2.5 % ethanedithiol as a scavenger of reactive cations generated. The cleavage mixture was filtered and precipitated in ice cold methyl t-butyl ether. The remaining resin was washed once with the cleavage reagent, filtered and combined with the previous fractions. The precipitates were collected after centrifugation, washed three times with ice cold methyl t-butyl ether and allowed to dry overnight at room temperature. The crude

peptides were dissolved in 15 % aqueous acetic acid and lyophilised for 2 days (-40 °C, 6 mbar).

Purification and Characterisation

The crude peptides were analysed by analytical LC-MS on a Quattro LC Mass Spectrometer from Micromass with a Hewlett-Packard HPLC instrument, model 1100 using analytical reverse-phase columns (column Alltech Hypersil PEP reverse-phase column, 100 Å, C_8, 5 μ (250 x 4.6 mm) 0 % → 50 % acetonitrile in 20 minutes. The separations were monitored at a wavelength of 215 nm for the amide bond absorbance with a flow rate of 1 mL/min. The crude peptides were purified by preparative reverse-phase HPLC (Gilson), monitored at 215 nm and eluted at a flow rate of 20 mL/min. The same mobile phase as stated for the LC-MS analysis of the crude peptides was used. The crude peptides were purified using an Alltech Hypersil PEP reverse-phase column, 100 Å, C_8, 8 μ (250 x 22 mm). They were eluted with 0 % → 50 % acetonitrile in 20 minutes. The analogues were greater than 95 % pure using high performance liquid chromatography (LC-MS) and had the expected amino acid analysis.

Various different gradients were applied for the elution of the peptides which were monitored at 215 nm. The organic phase, acetonitrile, and the aqueous phase both contain 0.1 % TFA and 3 % 1-propanol. The gradients and flow rates are listed below. The percentage indicates the proportion of the organic phase. 0 % → 50 % in 20 min, flow rate of 1 mL/min.

Abbreviations

MBHA, methylbenzhydrylamine; Fmoc, 9-fluorenylmethoxy-carbonyl; Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Abu, aminobutyric acid; Cys, cysteine; Gln, glutamine; Glu, glumatic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Trt, trityl; tBu, *tert*-butyl; Boc, butoxycarbonyl; Pybop, benzotriazol-1-yl-oxy-tris-pryrrolidino-phosphonium hexafluoro-phosphate; NMM, N-methyl morpholine; DCM, dichloromethane; DMF, dimethylformamide; TFA, trifluoroacetic acid; HPLC, high performance

liquid chromatography; LC-MS, liquid chromatography mass spectrometry; Å, Angström; AAA, amino acid analysis; DMSO, dimethyl sulfoxide; VEGF, vascular endothelial growth factor.

Reference may be made to the accompanying drawings. Briefly, Fig. 1 shows inhibition of VEGF radiolabelled ligand binding to porcine aortic endothelial cells (PAE) expressing only Neuropilin-1 (NP-1) by a dicyclo exon 7-derived peptide (number 3287), and no effect of a number of other related cyclic peptides. Fig. 2 shows selective inhibitory effects on PAE/NP-1 cells, but no effect on binding to cells (NAE/KDR) expressing only KDR (the other main VEGF receptor). In Fig. 1, F9 and D10 refer to two fractions of the same peptide preparation.

CLAIMS

1. A peptide having the amino acid sequence

SCKNTDSRCKARQLELNERTCRCDKPRR

or a fragment thereof that substantially retains NP-1 antagonist activity, in cyclic form.

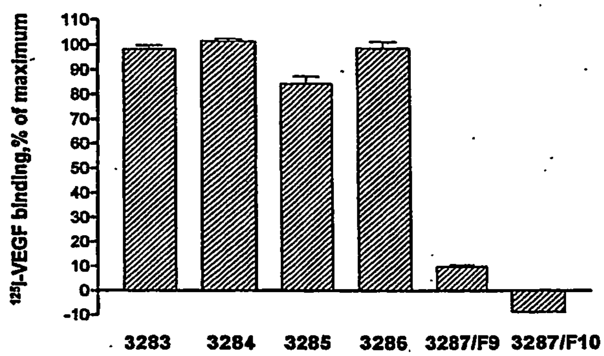
2. A peptide according to claim 1, for therapeutic use.

3. Use of a peptide according to claim 1, for the manufacture of a medicament for stimulating nerve repair.

4. Use of a peptide according to claim 1, for the manufacture of a medicament for the treatment of neurodegeneration.

Fig. 1

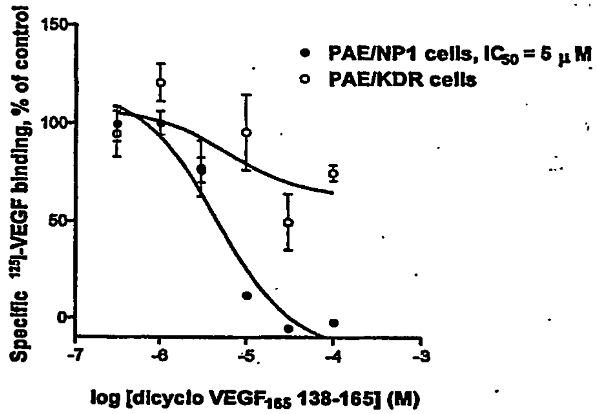
Effects of Cyclised VEGF Exon 7-Derived Peptides (100 μM) on ^{125}I-VEGF$_{165}$ Binding to PAE/NP1 Cells



3283, cyclo VEGF$_{189}$ (152-163) mutant: *CVQDPQT OKOSC*
3284, cyclo VEGF$_{189}$ (154-161) mutant: *CDPQTOKC*
3285, cyclo VEGF$_{189}$ (152-163) mutant: *YCVQDPQT OKOSCY*
3286, dicyclo VEGF$_{165}$ (111-138): ARQENPCGPC'SERRKHLFVQDPQTCKC'S
3287/F9, dicyclo VEGF$_{165}$ (138-165): SC'KNTDSRCKARQLELNERTC'RCDKPRR
3287/F10, dicyclo VEGF$_{165}$ (138-165): SC'KNTDSRCKARQLELNERTC'RCDKPRR

O = Aminobutyric acid

Fig. 2



150
100
50
0
-7
-6
-5
-4
-3
Specific 125I-VEGF binding, % of control
log [dicyclo VEGF165 138-165] (M)
PAE/NP1 cells, IC50 = 5 μM
PAE/KDR cells

PCT GB 2003 001375